

STUDIES ON THE SOURCE OF THE
LYTIC PRINCIPLE; AND ON THE
ORIGIN OF TRANSMISSIBLE
BACTERIAL AUTOLYSIS

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LYTIC PRINCIPLE; AND ON THE
ORIGIN OF TRANSMISSIBLE
BACTERIAL AUTOLYSIS

By
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I. INTRODUCTION

Several years ago d'Herelle¹ found the stools from patients convalescent from bacillary dysentery contained a filterable substance which was able to cause the lysis of broth cultures of *B. dysenteriae* Shiga. When a few drops of the autolysed culture was added to a fresh culture the same phenomenon was reproduced, and so on, in a continuous serial transmission of lytic action. Through these different passages the activity of the lytic property, instead of decreasing on dilution, increased and kept its lytic power for several years.

The continuous transmission of the lytic property occurred only when the transfer was made into young living cultures of the dysentery bacillus. Because of this, and for other reasons, d'Herelle concluded that the lytic agent was a filterable virus, parasitic on the dysentery bacillus. He gave the name "*Bacteriophagum intestinale*" to this assumed virus. However, he found later a similar lytic agent for *B. coli*, *B. typhosus*, and several other microorganisms. According to d'Herelle's view, there is but one bacteriophage, but this is adaptable to many, perhaps all, forms of bacteria.

In 1896 Hankin¹³ reported that the water of the Ganges and Jumna Rivers in India were bactericidal for bacteria, and particularly for the cholera vibrio. He ascribed this action to volatile substances present in the water, but d'Herelle concludes that this action was due to a bacteriophage.

Nicolle¹⁸ (1907) was probably the first to describe a bacteriolytic substance that may have been similar to the bacteriophage of d'Herelle. He reported that the filtrate of a broth culture of *B. subtilis* dissolved *B. coli*, pneumococci, *B. typhosus*

and cholera vibro. He demonstrated that broth cultures of pneumococci, *B. coli*, *B. dysenteriae* Shiga, and *B. typhosus* inoculated with *B. subtilis*, cleared. He did not investigate the question further.

Twort²⁴ (1915) observed in colonies of staphylococci, obtained while plating out glycerinated calf vaccine, transparent areas in which no cocci grew, or if they had grown, became disintegrated. When the transparent areas were touched with a sterile wire, and the wire drawn over the surface of an agar culture of normal staphylococci a clear and transparent path resulted after a few hours. He was able to demonstrate the passage of the lytic substance from the transparent areas through porcelain filters. The filtrates dissolved and killed most of the organisms in young staphylococcus broth cultures even in high dilutions. The lytic property could be transmitted through many tube generations as a result of adding the lysed culture to successive fresh staphylococcus cultures. d'Herelle^{1c} has not accepted the phenomenon described by Twort as an instance of the action of a bacteriophage although the identity of the two phenomena is now generally recognized.

Kabeshima¹⁴ (1920) who studied the lytic phenomenon believed that the activity of the lytic manifestation is caused by a catalyzer which is present in the glands of the intestines and which acts on a prodiastase in micro-organism thus forming a ferment which acts as a catalyzer for organisms of a new generation. He declines to accept d'Herelle's virus theory and shows that the properties of the lytic substance are more similar to those of an enzyme than those of a living organism.

The next important contribution on the lytic phenomenon was that of Bordet and Ciuca⁶ (1920) who were able to demonstrate that a lytic principle could be obtained without starting a stool filtrate.

Bordet and Ciuca isolated a lytic agent from a filtrate of the peritoneal exudate produced by repeated intraperitoneal injections of a guinea-pig

with *B. coli*. When this exudate was added to a normal broth culture of colon bacilli a lysis was produced which was also transmittable in series. They⁵ regard this phenomenon as one of body defense developed by the leucocytes, which impart to the organisms a hereditary property of producing a lytic ferment ("hereditary nutritive vitiation").

Bail² (1921) and Wollstein²⁵ (1921) were able to obtain a lytic agent from peritoneal exudates after the injection of Shiga bacilli, using the method of Bordet and Ciuca.

Kuttner¹⁵ (1923) succeeded in a similar undertaking when *B. typhosus* was used. d'Herelle¹, however, claims to have been unable to confirm the results of Bordet and Ciuca.

Debré and Haguénau⁸ (1920) found active bacteriophage in stools from cases of acute dysentery and enteric fever. However, they were unable to obtain lytic filtrates from the stools of healthy or ill infants.

Gildemeister¹⁰ (1921) was able to demonstrate the presence of a lytic principle in the stools during intestinal infections active against bacilli of the Colon-Typhoid-Dysentery group.

Wollstein²⁵ (1921) prepared filtrates from the stools of 23 infants including six which had gastrointestinal disturbances, and one convalescent from Shiga dysentery. A lytic filtrate was obtained from only one and that a fatal case of *B. coli* peritonitis. The filtrate was active against colon and Shiga bacilli.

Rimpau²² (1921) obtained a lytic filtrate from the stool of a healthy individual in the vicinity of a case of typhoid active against dysentery bacilli but not against typhoid.

Kuttner¹⁵ (1923) was able to obtain a bacteriophage from a typhoid convalescent active against typhoid, Flexner and Shiga bacilli. She found that normal glycerinated tissue extracts of the small intestine and liver of a guinea pig were bacteriolytic

for *B. typhosus* and that this lytic agent could be transmitted in series. However, she states, "The presence of lytic agents in these tissues active against typhoid bacilli is, however, extremely rare."

Grati^{all} (1921) obtained from vaccinia a staphylococcus bacteriophage that could be transmitted in series. Callow⁷ (1922) demonstrated the presence of a bacteriophage transmittable in series in the pus of 16 staphylococcoses.

The foregoing references, representing only a portion of those dealing with the isolation of the bacteriophage, are sufficient to illustrate its variety of source, and the need of further concise knowledge on this point. The present study therefore deals mainly with attempts to isolate the lytic agent from various sources, and to ascertain how and under what conditions it is generated.

II. ATTEMPTED ISOLATION OF THE LYTIC AGENT FROM NORMAL ANIMALS

1. Hog Intestines

In an attempt to isolate a lytic principle from hog intestinal contents, six hogs were examined. Sections of the small intestine near the junction of the large were obtained from the slaughter house.

The content of each section was removed and then washed with sterile physiological salt solution. After slitting open and fastening down upon sterile absorbent paper, the inner surface was thoroughly scraped and ten drops of the mucosa placed in flasks containing 50 c.c. of sterile plain meat infusion broth with a pH of 8.0. The six flasks containing the inoculated broths were then incubated at 35 C. for 24 hours, at the end of which time a marked turbidity was present in all the tubes.

The six broths were next diluted by taking five c.c. of each and adding it to 20 c.c. of sterile

plain meat infusion broth adjusted to a pH of 8.0. These various dilutions were then filtered through a sterile Berkefeld N filter and labeled Hog. No. 1.; Hog. No. 2.; etc.

Each of the six filtrates was diluted, according to the following method and then tested for inhibition against the organisms indicated in Table I:

To 0.5 c.c. of each filtrate was added 4.5 c.c. meat infusion broth. Each diluted broth was then inoculated with 0.01 c.c. of a 12-hour broth culture of the organisms enumerated in Table I. The inoculated tubes were incubated at 35 C. for 24 hours when the final readings were made. The degree of inhibition was compared with the broth control tubes of the same organisms.

From Table I it is apparent that an inhibiting action was present in the filtrates of Hog 5 and Hog 6 causing a slight lag in the growth of *B. dysenteriae*-Flexner. A trace of inhibition was observed in the case of the Kruse strain with the same filtrates.

In order to confirm the lytic action in filtrates 5 and 6 and to ascertain whether the inhibitive action noted was transmissible in series, the following tests were carried out:

Two tubes of five c.c. each of the original filtrates Hogs 5 and 6 were inoculated as follows:

To tube No. 1 from Hog 5 was added 0.01 c.c. of a 12-hour broth culture of *B. dysenteriae*-Flexner, producing no recognizable turbidity.

To tube No. 2 from Hog 5 was added 0.1 c.c. of the same broth culture, producing a grade 1+ turbidity.

To tube No. 3 from Hog 6 was added 0.01 c.c. of the same broth culture, producing no recognizable turbidity.

Table I
The Action of the Hog Intestine Filtrates Against
Organisms of the Intestinal Group

Organism	Age of Culture	Amount of Culture Inoculated	Results					
			Hog 1	Hog 2	Hog 3	Hog 4	Hog 5	Hog 6
B. Coli	Hrs. 12	c.c. 0.01	None	None	None	None	None	None
B. typhosus-Eberth	12	0.01	"	"	"	"	"	"
B. " -Gay	12	0.01	"	"	"	"	"	"
B. para typhosus "A"	12	0.01	"	"	"	"	"	"
B. " "B"	12	0.01	"	"	"	"	"	"
B. Enteritidis	12	0.01	"	"	"	"	"	"
B. dysenteriae-Flexner	12	0.01	"	"	"	"	Slight	Slight
B. dysenteriae-Kruse	12	0.01	"	"	"	"	Trace	Trace
B. " -Shiga	12	0.01	"	"	"	"	None	None
B. " -Strong	12	0.01	"	"	"	"	"	"
B. cholerae suis	12	0.01	"	"	"	"	"	"

To tube No. 4 from Hog 6 was added 0.1 c.c. of the same broth culture, producing a grade 1+ turbidity.

Tube No. 5 (control) contained 5 c.c. sterile meat infusion broth received 0.01 c.c. of the same broth culture which produced no recognizable turbidity.

To tube No. 6 (control) containing 5 c.c. sterile meat infusion broth; was added 0.1 c.c. of the same broth culture, producing a grade 1+ turbidity.¹

After incubating at 37 C., for eight hours tubes 1 and 3 showed a trace of cloudiness, while tube 5 Control showed a grade 1+ turbidity. However, in tubes 2 and 4 there was an increase in turbidity, not as distinct as in tubes 1 and 3. Control tube 6 showed a grade of 2+ turbidity.

The tubes were again incubated up to 24 hours. The results for both readings are expressed in Table II.

Table II

Showing the Reaction of the Filtrates 5 and 6
Further Inoculation with Young Cultures
of *B. dysenteriae*-Flexner

Time of Observation	Hog 5		Hog 6	
	Tube 1	Tube 2	Tube 3	Tube 4
8 hours	Trace	+	Trace	+
24 hours	clēar	trace	clēar	trace

¹In the present work the following symbols are used:

- ++++ maximum cloudiness
- +++ moderate "
- ++ slight "
- + least possible observed
- trace-barely perceptible
- clear, no cloudiness.

It was thus apparent that a lytic agent was present in the intestines from Hogs 5 and 6. The lytic phenomenon was further studied in an attempt to demonstrate that the lytic principle was transmissible in series.

The corresponding tubes from the preceding tests were pooled and filtered through a Berkefeld N candle. Five c.c. of each the filtrates 5 and 6 were diluted with 20 c.c. of meat infusion broth adjusted to pH 8.0.

To tube No. 1, containing 5 c.c. of the filtrate 5, was added 0.1 c.c. of a 12-hour broth culture of Flexner bacilli.

To tube No. 2 containing 0.5 c.c. of the filtrate 5 and 4.5 c.c. sterile plain broth, was added 1.0 c.c. of a 1.2-hour broth culture of Flexner bacilli.

To tube No. 3, containing 5 c.c. of the filtrate 6, was added 0.1 c.c. of the same broth culture.

To tube No. 4, containing 0.5 c.c. of the filtrate 6 and 4.5 c.c. sterile plain broth, was added 1.0 c.c. of the same broth culture. These tubes were incubated for 48 hours. The results, which are presented in Table III show that filtrates 5 and 6 lysed young Flexner bacilli.

After the tubes had been incubated for six hours, two agar slants were streaked respectively with four loops from tubes 1 and 3. After 24 hours incubation the slant from filtrate 5 showed no lytic areas nor any irregular colonies, but a normal growth. However, the slant from filtrate 6, though containing no lytic areas, showed a few irregular, lytic colonies.

A transmission series was next attempted from Tubes 1 and 3; following a 12-hour incubation, when complete lysis had taken place. One drop from

Table III

Showing the Action of the Hog Filtrates
5 and 6 Against *B. dysenteriae*-Flexner

Time of Observation	Hog 5		Hog 6	
	Tube 1	Tube 2	Tube 3	Tube 4
start	trace	++++	trace	++++
6 hours	+	++++	+	++++
12 "	-	++	-	++
18 "	-	+	-	+
24 "	-	trace	-	trace
36 "	-	trace	-	trace
48 "	-	+	-	+

Tube 1 was added to a young broth culture of Flexner bacilli, and this culture lysed. In this manner several successive passages were made, each time a drop of the previously lysed culture being introduced into a young broth culture. Filtrate 5 ceased to lyse upon the third passage while filtrate 6, carried through an identical series, ceased at the fourth passage. From these results it was manifested that filtrates 5 and 6 apparently contained lytic substance that was capable of being transmitted in series. Undoubtedly the series could have been carried further had a greater amount of lysed culture been added at each inoculation.

An Attempt to Increase the Lytic Power of Filtrates 5 and 6 by Feeding

All tubes of the filtrates 5 and 6 from the last test were pooled and filtered. Ten c.c. of each filtrate was inoculated with 0.5 c.c. of a 12-hour broth culture of Flexner bacilli, resulting in a grade 1+ turbidity. The tubes were then incubated

at 37 C. for 12 hours at which time complete lysis had occurred in both tubes. The two filtrates were again fed 0.5 c.c. of a 12-hour broth culture, giving a grade 1+ turbidity. Upon a further incubation period of 12 hours complete lysis had again taken place. The filtrates were fed for a third time with 0.5 c.c. of a 12-hour broth culture of Flexner bacilli giving a grade 1+ turbidity. The inoculated broth filtrates were again incubated for 12 hours, when it was noted that a partial lysis had taken place in both filtrates. However, upon further incubation there was no further inhibition. It seemed probable that the turbidity was due to the growth of a resistant strain of the dysentery bacillus. The broths were next filtered and five c.c. of each filtrate 5 and 6 were added to 25 c.c. of meat infusion broth and again fed 0.1 c.c. of a 12-hour broth culture of Flexner bacilli and incubated. Twelve hours later they were again fed, but the amount was increased to 0.5 c.c. Twenty-four hours later the broths were clear and were fed 0.5 c.c. of a 12-hour broth culture of B. Flexner. Upon further incubation of 24 hours they again cleared. Each broth tube was then fed 1.0 c.c. of a broth culture, giving a grade ++ clouding. It was only after 48 hours' incubation that the tubes again became clear. They were then again fed 1.0 c.c. of a 12-hour broth culture of Flexner bacilli. Following this last feeding neither of the tubes cleared; then they were both filtered.

It may be noted that the method of increasing the lytic power used in the above tests differs from that used by d'Herelle^{1b} and others in that the young cultures are added to the lytic filtrates. Following each filtration the dilution was increased about four to one. The amount of young broth culture was increased at each feeding. It was evident that the lytic power increased as the dilutions from the original tube increased; moreover that there was an increase in the amount of young broth culture consumed at each feeding.

The Effect of the Lytic Agent
on the Organisms

A study of the effect of the lytic agent upon the cultural and morphological characters of *B. dysenteriae*-Flexner was conducted.

Tube 1 contained 0.5 c.c. of filtrate 5 from the preceding test and 10 c.c. of meat infusion broth adjusted to pH 8.0.

Tube 2 (control) contained 10 c.c. of plain broth as used in Tube 1.

Each of the above tubes was inoculated with 0.1 c.c. of a 12-hour broth culture of Flexner bacilli. The tubes were incubated for 12 hours when the first observation was made. Tube 1 was clear; apparently the bacilli had been inhibited or lysed. After another incubation of 12 hours, making a total of 24 hours, Tube 1 possessed a grade 2+ turbidity while the control was a grade 4+.

In addition, at the beginning of the test four loops from each of the tubes were streaked over the surface of two agar slants. Following 12 hours' incubation, the slants showed a very thin scanty growth, having no lytic areas or colonies. The Control slants appeared to be normal. When these same slants were examined after 24 hours it was noted that the controls had a normal uniform growth over the surface of the slants. However, those streaked from filtrate 5 showed a thin, scanty growth, but no recognizable lytic areas. There appeared to be two different types of colony: One that was round and moist with a smooth entire edge and a slightly denser central area; the second, which was very irregular in shape and size, possessing punctured or indented areas in its thin irregular edges. This type was granular and there was no raised center, but a shrunken central area.

A number of both sorts of the isolated colonies were removed and planted in meat infusion broth. The regular type grew rapidly and profusely

causing the broth to become turbid. The irregular type grew very slowly, and a granular mass gathered at the bottom of the tubes leaving a clear upper layer.

A set of agar slants seeded at the end of 24 hours had the same types of colony, but they were more scattered and a larger proportion were of the resistant type.

Filtrate 6 gave results comparable to filtrate 5. From the study of filtrates 5 and 6 it was concluded that when Flexner bacilli were exposed to these filtrates there was formed a new resistant type of colony which occurred along with the sensitive type, but which increased in number with the ageing of the culture submitted to the lytic action.

Next, an attempt was made to test the inhibition titer of filtrates 5 and 6 against various organisms of the Colon-typhoid and Dysentery groups.

Five c.c. of a 1 - 50 dilution of filtrate 5 lytic principle from the preceding experiment was placed in 10 sterile test tubes. Each tube was inoculated with 0.01 c.c. of a 12-hour broth culture of the organism selected and mentioned in Table IV. The inoculated tubes were incubated and readings taken at 12 hours; 24 hours and 48 hours. The degree of inhibition was compared with control tubes, inoculated, incubated and read under similar conditions.

From the data presented in Table IV it appeared that the filtrate 5 gave complete inhibition of the Flexner bacilli and a slight inhibition towards Kruse, Shiga and Strong bacilli. No inhibition was observed against the other organisms tested. Filtrate 6 gave essentially the same results, except that there was a trace of inhibition against *B. paratyphosus* "A" and "B".

In this case an attempt was made to ascertain the maximum titer of filtrates 5 and 6 against Flexner bacilli before and subsequent to increasing the lytic power.

Table IV

Showing the Inhibition Titer of Filtrates
5 and 6 Against Members of the Colon-Typhoid
and Dysentery Groups

Organism	Results*	
	Filtrate 5	Filtrate 6
B. Coli	None	None
B. typhosus-Eberth	"	"
B. " -Gay	"	"
B. paratyphosus "A"	"	Trace
B. " "B"	"	"
B. Enteritidis	"	None
B. dysenteriae-Flexner	Complete	Complete
B. " -Kruse	Slight	Slight
B. " -Shiga	"	"
B. " -Strong	"	"

* The readings tabulated are for 12 hours. Those for 24 and 48 hours were the same.

One c.c. of each filtrate was first diluted with 9 c.c. of sterile plain broth, thus making a 10^{-1} dilution. From this tube a series of dilutions were made, each increasing by 10. Each tube was then inoculated with one (2 mm) loop of a 12-hour broth culture of Flexner. Controls on the broth, organism and filtrate were included in the set-up. Final readings were taken at the end of 48 hours of incubation at 35 C.

The same method of procedure was carried out for filtrate 6 which was carried through identical experiments. The results of these tests are presented in Table V.

Table V

Showing the Maximum Titers of Filtrates 5 and 6 for Dysentery Flexner

Strain	Results									
	Broth Control	Filtrate Control	B. Flexner Control	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸
Hog 5 (before feeding)	-	-	++++	-	-	++++	++++	++++	++++	++++
Hog 5 (after feeding)	-	-	++++	-	-	-	-	++++	++++	++++
Hog 6 (before feeding)	-	-	++++	-	-	-	++++	++++	++++	++++
Hog 6 (after feeding)	-	-	++++	-	-	-	-	-	++++	++++

Note: (-) indicates complete inhibition, (++++), growth, or no inhibition.

From the data presented in Table V it was concluded that the maximum titer of filtrate 5 before feeding was 10^{-3} and that it was increased to 10^{-5} by two successive feedings of young broth culture.

Filtrate 6 was increased in lytic value from 10^{-4} to 10^{-6} using an identical method and the same number of feedings.

After this test the two strains 5 and 6 were fed various but increasing amounts of a 12-hour broth culture of Flexner bacilli over a period of 20 months with frequent filtration. The following method was employed. After each filtration two c.c. of filtrate was added to 25 c.c. sterile meat infusion broth adjusted to pH 8.0. The diluted filtrates were fed increasing amounts of young broth cultures, 0.1 c.c., 0.25 c.c., 0.5 c.c., 1.0 c.c. and 1.5 c.c., until the culture became permanently clouded with secondary growth, when the turbid broths were filtered. These feedings were performed 18 times with filtrate 5 and 19 times with filtrate 6 over the above mentioned time.

Titration of the lytic power were performed following several of the filtrations, the method of procedure being the same as previously described. The results are recorded in Table VI.

From the data presented in Table VI it is clear that the inhibition titer of filtrate 5 was increased from 10^{-3} to 10^{-7} against Flexner bacilli as a result of feeding young broth cultures, with intermediate filtration. Likewise, the titer of filtrate 6 was increased from 10^{-4} to 10^{-7} by the same method.

Filtrate 5 reached its maximum power following the eleventh feeding and remained constant at least through the eighteenth, while filtrate 6 reached its maximum at the ninth and did not increase until after the fifteenth titration. Apparently, beyond a certain number of feedings, for each filtrate, the inhibition titer is not significantly increased by further feedings.

Table VI

Summary of the Inhibition Titrations Performed on
Filtrates 5 and 6 Against *B. dysenteriae*-Flexner

Strain	Titration	Broth Control	L.P. Control	B. Flexner Control	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹
Hog 5.	1	-	-	+	-	+	+	+	+	+	+
	2	-	-	+	-	-	-	+	+	+	+
	7	-	-	+	-	-	-	+	+	+	+
	9	-	-	+	-	-	-	-	+	+	+
	11	-	-	+	-	-	-	-	+	+	+
	13	-	-	+	-	-	-	-	+	+	+
	15	-	-	+	-	-	-	-	+	+	+
	18	-	-	+	-	-	-	-	+	+	+
Hog 6.	1	-	-	+	-	-	+	+	+	+	+
	2	-	-	+	-	-	-	-	+	+	+
	7	-	-	+	-	-	-	-	+	+	+
	9	-	-	+	-	-	-	-	+	+	+
	11	-	-	+	-	-	-	-	+	+	+
	13	-	-	+	-	-	-	-	+	+	+
	15	-	-	+	-	-	-	-	+	+	+
	19	-	-	+	-	-	-	-	+	-	+

2. Rabbit Intestines

After a lytic principle had been obtained from hog intestines attempts were made to obtain the same from intestines of other animals. The next tests were made with material from rabbits.

Twenty-four normal rabbits which had been bled to death for the purpose of securing blood were examined and attempts made to obtain a lytic agent from the intestinal contents and the intestinal mucosa.

The same portions of the intestine were used as in the work with hogs. The content was removed and the inside of the section washed out with 25 c.c. of meat infusion broth; this broth was used for the tests. The section was slit and the same technique employed as with the hog intestines.

The various filtrates were then tested against *B. dysenteriae* Shiga in a series of three experiments. In the first, the original filtrate was tested. The various filtrates were then fed very small amounts of young broth cultures, filtered and again tested. The filtrates were next fed a second time, filtered and tested again as previously described.

In addition, an inhibition series of three tubes were made from each of the inoculated filtrates. One loop from the original filtrate was added to a tube of sterile broth, then one loop of the latter added to another tube of sterile broth and so on. No inhibition was noted of the series.

It was concluded from the results obtained that no lytic agent was present in the normal rabbit intestines active against *Shiga bacilli*, at least after two series of feeding and filtration.

3. Guinea-Pig Intestines

A similar attempt was made to isolate a lytic agent from guinea pigs. The intestinal content

and intestinal tissue of 12 normal animals were used. Identical methods of procedure were carried out as in the work with rabbits.

The results of three consecutive tests showed that no lytic agent appeared to be present in the filtrates.

Agar slants were streaked from each tube of the third test, but no lytic areas were obtained.

No inhibition was shown in any tube of the inhibition series.

From the results of this work it was concluded that there was no lytic or inhibiting agent present in the intestinal content and intestinal tissue of the normal guinea pigs active against the Shiga bacillus.

III. ATTEMPTS TO ISOLATE THE LYTIC PRINCIPLE FROM EMBRYONIC TISSUE

In recent years, there have appeared a number of articles relating to the bactericidal action of living tissue, but none have dealt directly with the lytic principle. Smyth^{23a} for example noted that plasma from 14-day chick embryos possessed a marked bactericidal action on *B. typhosus*, a slight action on *B. dysenteriae* and no action on *B. coli*. However, he made no attempt to transmit this germicidal substance from one tube to another.

1. Chick Embryonic Tissue

It seemed of interest therefore to attempt the isolation of a lytic agent from embryonic tissue. In the first series of tests, chick embryos were used.

Nine chick embryos were removed from eggs on the twentieth day. These live embryos were handled under aseptic conditions. They were cut into small pieces about the size of a pea and ground in a large test glass with sterile sand by means of a heavy sterile glass rod. One hundred c.c. of sterile meat infusion broth were added to each ground embryo and well-triturated suspensions were placed in sterile flasks and inoculated with 0.5 c.c. of 12-hour broth culture of *B. dysenteriae*-Shiga and incubated at 35 C. for 72 hours.

To five c.c. samples of each of the inoculated suspensions were added 20 c.c. of plain broth. The diluted suspensions were then filtered through pumice and then through Berkefeld N filters. The filtrates were then tested for sterility and inhibition tests performed against Shiga bacilli according to the usual technique. Five c.c. of each the filtrates were placed in sterile test tubes and each was inoculated with 0.01 c.c. of a 12-hour broth culture of *B. dysenteriae*-Shiga. The tubes were incubated for 24 hours when the final results were recorded. No lytic agent was demonstrated in any of the original filtrates.

Further tests were made to confirm the absence of lytic agent in chick embryonic tissue; also to ascertain whether it could be produced by prolonged contact with cultures of bacteria.

The tubes from the preceding experiment were diluted with 20 c.c. meat infusion broth adjusted to pH 8.0. The diluted broths were filtered through a Berkefeld N candle. A method of procedure identical to that used in the preceding experiment was employed. The tubes were incubated as in the preceding experiment. The results showed that continued contact with the organisms yield no lytic agent.

The inoculated filtrates from the last experiment were next diluted with 20 c.c. meat infusion broth and filtered through a Berkefeld N candle. The preceding experiment was repeated, but no inhibition was noted in any of the tubes.

An inhibition series of three tubes was made from each of the inoculated filtrates. No inhibition was noted.

In addition, agar slants were streaked from each of the inoculated filtrates. Normal growths result with no lytic areas present.

It was concluded from these results that there was no lytic agent present in the filtrates derived from the embryonic tissue of chicks, either at the beginning or after prolonged contact with Shiga cultures.

For a confirmatory test a second set of nine living embryos was obtained and the same method followed in detail. The results obtained showed that here also there was no sign of lytic action. A third set of seven living chick embryos gave the same negative result. One may therefore conclude from the results of the 9 tests that the lytic agent is not commonly present in chick embryonic tissue and that it is not commonly developed from contact of embryonic tissue with bacterial cells in the case of the most sensitive organism studied, *B. dysenteriae*-Shiga.

2. Rabbit Embryonic Tissue

Other tests were performed upon rabbit embryonic tissue. Four sets of living rabbit embryos were taken from the uterus of normal pregnant rabbits. The embryos were within about two days of term. They were cut in pieces about the size of a pea, ground with sterile sand as in previous cases. A suspension was made with 50 c.c. of sterile meat infusion broth adjusted to pH 8.0. The same method

of testing was used as with the chick embryos.

The results from these tests embracing 34 embryos showed that no lytic action was present in the original embryonic tissue filtrates; moreover, that none appeared to be formed from contact of living rabbit embryonic tissue with bacterial cells in the case of Shiga dysentery cultures.

3. Rat Embryonic Tissue

Similar tests were made upon the embryonic tissue of rate.

Four sets of living rat embryos were obtained from normal females. The embryos were removed from the embryonic sac about one day preceding term. The same method of procedure was used as with the chick and rabbit embryos.

The results from these tests, embracing 24 embryos, showed that no lytic action against B. dysenteriae-Shiga was to be observed either in the original tissue filtrates, or in filtrates in prolonged contact with the bacterial cells.

4. Guinea-Pig Embryonic Tissue

To conclude this phase of the work tests were performed upon embryonic tissue of guinea pigs. Four sets of living embryos were used freshly removed from the uterus. The same method of procedure was employed as previously described.

The results embracing the study of 8 embryos showed that no lytic agent was present in the original tissue filtrates and that none was formed by prolonged contact of the tissue with bacterial cells in the case of Shiga dysentery.

From the results obtained from the study of chick, rabbit, rat and guinea-pig embryonic tissue it appears safe to conclude that there is present in embryonic tissue no substance which has the power

to incite the lytic process in Shiga dysentery cultures. Moreover, that the inhibitive action of embryonic tissue upon bacterial growth, as observed by Smyth^{23a} and others, does not rest upon the same factors which initiate or support a transmissible bacterial autolysis.

IV. ISOLATION OF THE LYTIC AGENT FROM INFECTED TISSUE

Since success was not attained in the attempt to obtain lytic agent from normal intestinal tissue, embryonic tissue, or their combination with bacterial cells, it was next attempted to isolate the lytic agent from tissues of infected animals.

1. Animals Inoculated with *B. dysenteriae*-Flexner

Sections of the small intestines were obtained from four rabbits that had been, or were being, immunized against *B. dysenteriae*-Flexner. This material was employed in attempts to obtain a lytic principle from the content and from the mucosa. The intestinal content was removed from each of the sections then washed out with 50 c.c. of sterile meat infusion broth adjusted to pH 8.0. The washings were placed in sterile flasks. The intestinal sections were slit and the mucosa thoroughly scraped. The sections were then cut into small pieces and ground with sterile sand. To each plastic mass was added 50 c.c. of sterile meat infusion broth. These broth suspensions were then inoculated with 0.5 c.c. of a 12-hour broth culture of Flexner bacilli and incubated for 72 hours at 35 C.

Five c.c. of the inoculated broth was then removed from the flask and added to 20 c.c. of meat

infusion broth. Each sample was then filtered through a Berkefeld N filter and each filtrate tested against the following strains of dysentery bacilli: (1) Flexner, (2) Kruse, (3) Strong, and (4) Shiga, through three series of dilutions and filtrations as described for the tests with normal rabbit intestine.

The results of the tests failed to reveal the presence of any inhibiting agent in any of the filtrates from either the intestinal mucosa or the content. Agar slants inoculated with four loops from the broth tubes after 24-hour incubation gave normal growth of the test organism.

2. Rabbit Immunized with Human Blood Serum

In another case, however, the author was able to obtain a lytic principle from the intestine of a rabbit which was being immunized against human blood serum. This rabbit had received three intravenous injections on alternate days. The second day following the last injection the animal gave birth to 16 young and died about four hours later. The necropsy showed only hemorrhage from blood vessels in the abdominal cavity.

Several small incisions were made in the small and large intestine and 10 drops of the thick mucus taken up with a sterile Pasteur pipette. This material was added to 25 c.c. of meat infusion broth and incubated for 24 hours at 35 C. The inoculated medium became slightly turbid. The broth was then filtered and divided into two portions. An inhibition test was then conducted against Shiga bacilli.

Tube 1 containing 10 c.c. of the filtrate was inoculated with one loop of a 12-hour broth culture of Shiga.

Tube 2 containing 10 c.c. of the filtrate served as control.

Tube 3 containing 10 c.c. broth was inoculated with Shiga.

The tubes were incubated for 24 hours at 35 C. At the end of this time as shown in Table VII Tube 1 showed complete inhibition, while the Shiga control showed normal growth. The filtrate control remained sterile.

Table VII

Showing the Inhibitive Action of Filtrate of Intestinal Mucosa of Immunized Rabbit

Time	Tube 1	Tube 2	Tube 3
6 hrs.	+ growth	No growth	+ growth
12 "	+ "	No "	++ "
24 "	Complete Inhibition	Sterile	++++ " (Normal)

Agar slants streaked from Tubes 1, 2 and 3 after 12 hours' incubation showed in No. 1 many scattered irregular and some normal colonies; in No. 2 no growth; in No. 3 a normal growth.

Two types of colonies were obtained and these were studied in detail.

One was a typical, moist round colony showing a smooth margin with a denser center. The other type was irregular in shape and size, showed a depressed central area and had a granular appearance. The round or regular colonies when transplanted in broth produced normal and abundant growth, while the irregular type of colony grew slowly, gradually settling to the bottom of the tube leaving the upper stratum clear or very slightly turbid.

Since it thus appeared that an inhibitive agent was present in the filtrate an attempt was

made to increase its potency.

A sample of the filtrate from the preceding test was diluted with an equal volume of infusion broth adjusted to pH 8.0. The broth was inoculated with a 12-hour broth culture of Shiga bacilli and incubated. At intervals thereafter additional culture was added. The results are shown in Table VIII.

Table VIII

Showing Details of the Test to Increase Potency of the Rabbit Filtrate by Feeding Dysentery Culture

Date	Amount of Culture fed	Incubated at 35 C	Results
1922			
July 14	0.01 c.c.	24 hours	Complete lysis
" 15	0.05 c.c.	24 "	" "
" 16	0.05 c.c.	24 "	" "
" 17	0.10 c.c.	24 "	" "
" 18	0.10 c.c.	24 "	" "
" 19	0.1 c.c.	24 "	" "
" 20	0.5 c.c.	24 "	" "
" 21	0.5 c.c.	24 "	" "
" 22	1.0 c.c.	24 "	" "
" 23	1.0 c.c.	24 "	" "
" 24	2.0 c.c.	24 "	Partial lysis
" 25		48 "	Complete lysis
" 26	3.0 c.c.	24 "	Partial lysis
" 27		48 "	No change
" 28		72 "	Turbid
" 29		96 "	No change

From the results reported in Table VIII it appears that there was present an inhibiting agent active for Shiga bacilli and which apparently could be increased in potency by the method of feeding.

The inoculated tube which had been fed at intervals over a period of 14 days was then filtered,

Table IX

Titration of the Inhibition Value of the Rabbit Filtrate

Time	Set-up					Results *				
	Broth Control	L.P. Control	B. Shiga Control	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Start	-	-	-	-	-	-	-	-	-	-
1 hr.	-	-	-	-	-	-	-	-	-	-
3 "	-	-	trace	-	-	-	-	trace	trace	trace
6 "	-	-	+	-	-	-	-	+	+	+
9 "	-	-	++	-	-	-	-	+	++	++
12 "	-	-	+++	-	-	-	trace	+	++	+++
24 "	-	-	++++	-	-	-	-	++	++++	++++
48 "	-	-	++++	-	-	-	-	+++	++++	++++
72 "	-	-	++++	-	-	-	-	++++	++++	++++

*The degree of inhibition is reported: - complete inhibition;

+ slight; ++ and +++ intermediate graduations; ++++ no inhibition.

after which an inhibition was performed.

A series of seven dilutions up to 10^{-7} was made. Each tube of the series was inoculated with one loop of a 12-hour broth culture of Shiga bacilli. The tubes of the set-up were then incubated at 37 C. and readings taken after the intervals noted in Table IX which presents the details of the test.

From the data presented in Table IX it appears that the inhibition titer for the Rabbit filtrate against Shiga bacilli was 10^{-4} .

An attempt was now made to increase this titer by the following method:

Two c.c. from the last filtration was added to 20 c.c. of infusion broth and fed at various intervals 0.1 c.c., 0.5 c.c., 1.0 c.c., 1.5 c.c., 2.0 c.c., etc., of a 12-hour broth culture of Shiga bacilli. The broth was not fed again until complete lysis had taken place. As soon as lysis did not take place the inoculated broth was filtered.

Over a period of 18 months, 17 such feedings and filtrations were performed. Titrations for the inhibition power were made following some, but not all, of the filtrations. The results are expressed in Table X.

In Table X it is shown that the feeding increased the inhibition power of the strain from 10^{-4} to 10^{-8} . The increase was gradual. Moreover, it is clear that the feedings from the 14th to the 18th, with the exception of No. 17, failed to raise the titer above 10^{-8} . This then probably represents the maximum titer obtainable with this strain of bacteriophage for the Shiga dysentery bacillus.

Table X

Summary of the Various Titrations Performed Upon the Rabbit
Lytic Principle Against *B. dysenteriae*-Shiga During the
Course of Feeding

Titration	Set-up				Results							
	Broth Control	L.P. Control	B. Shiga Control	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	
1	-	-	+	-	-	+	+	+	+	0	0	
2	-	-	+	-	-	+	+	+	+	0	0	
4	-	-	+	-	-	-	+	+	+	0	0	
11	-	-	+	-	-	-	-	+	+	0	0	
12	-	-	+	-	-	-	-	+	+	+	0	
13	-	-	+	-	-	-	-	-	+	+	+	
14	-	-	+	-	-	-	-	-	-	+	+	
15	-	-	+	-	-	-	-	-	-	+	+	
16	-	-	+	-	-	-	-	-	-	+	+	
17	-	-	+	-	-	-	-	-	-	-	+	
18	-	-	+	-	-	-	-	-	-	+	+	

- = complete inhibition, + = no inhibition, 0 = no test made.

3. Spontaneous Rabbit Abscess

A rabbit that had developed an abscess about the size of a hen's egg on its neck was anaesthetized. Upon opening the abscess a thick, somewhat viscous, creamy pus was obtained. Microscopical examination of the pus revealed a small gram-negative cocco-bacillus. Cultures were made of the pus and tissue in meat infusion broth and on plain agar and blood agar plates.

The broth tubes became very turbid. The growth on the blood agar was more luxuriant than that on plain agar. There were two types of colonies on the plate: one, round with regular edges, thick, opaque and moist. The other was very irregular in shape and size and translucent. Some possessed a faint bluish hue and granular. The regular colony grew diffusely in plain broth. Its virulence for rabbits was nil.

The organism concerned was identified as a member of the *Pasteurella* group, probably identical with the cocco-bacillus of rabbit abscess described by Piorkowski, usually termed *Bacillus cuniculicida*. It is probably a member of the same group studied by d'Herelle^{1c} in the case of barbone of the East Indian buffalo.

Agar slant cultures were made from both types of colony. The regular type produced a normal luxuriant growth. However, the irregular type produced a thin, granulous growth on the surface with exception of the extreme upper portion of the slant. In some instances a secondary growth appeared over the lytic site. The author was able to carry this peculiar suicidal culture through 75 transplants by streaking the culture on agar at 12-hour intervals or less. At the end of that period the culture perished in a rapid lysis, from which only the resistant type could be recovered, thus terminating the work on the sensitive type.

Four of the original broth tubes were filtered through pumice and then a Berkefeld N candle.

An inhibition test was performed with the filtrate against the organisms listed in Table XI using the same method of procedure earlier described. Unfortunately, at this time, it was not possible to test the filtrate on the original culture although other members of the Pasteurella group were used, including Piorkowski's original culture.

Table XI

Showing the Results of an Attempt
to Isolate a Lytic Agent from a Rabbit Abscess

Organism	Age of Culture Inocu- lated	Amount of Culture	Results	
			Rabbit #75 Filtrate	Organism Control
	hr.	c.c.		
<i>B.cholerae suis</i>	12	0.01	+	+
<i>B.gallinarum</i>	12	0.01	+	+
<i>B.avisepticus</i>	12	0.01	+	+
<i>B.cavisepticus</i>	12	0.01	+	+
<i>B.typhosus</i> - Eberth	12	0.01	+	+
<i>B.dysenteriae</i> - Shiga	12	0.01	-	+
Cocco bacillus of rabbit abscess: (Piorkowski)	12	0.01	+	+
<i>B. coli</i>	12	0.01	+	+

- means complete inhibition, and + = no inhibition.

Curiously enough no inhibitive action of the abscess filtrate was shown against the organisms of the Hemorrhagic Septicemic Group, or Colon-Typhoid Group, but lytic action against the Shiga bacilli was demonstrated in a dilution of 10^{-4} .

After three feedings with Shiga bacilli the filtrate was again tested against the Hemorrhagic Septicemic Group and also the homologous resistant strain. No inhibition was noted.

In addition, agar slants inoculated from the tubes of the set-up showed normal growth of all the test organisms.

The filtrate was then fed Shiga bacilli three more times and filtered, after which a second titration for its inhibitive power was performed against a sensitive B. Shiga. A titer of 10^{-6} was obtained.

In the course of this six attempts were made to produce a sensitive strain from the resistant organism isolated from the original abscess culture but without success. This seems to be a case similar to that of the "Suicide Cultures" first mentioned by Haffkine^{1c} and observed in this laboratory on several occasions.

V. THE POSSIBLE ORIGIN OF THE LYTIC AGENT IN THE BACTERIA THEMSELVES

Several publications have appeared within the last few years to the effect that old bacterial cultures contain the lytic agent. Bail² (1921), Bergstrand⁴ (1922), Kuttner¹⁵ (1923), Pico²⁰ (1922), and others^{3, 10, 16, 19, 21}, believe they have been able to demonstrate its presence in old cultures.

The organisms used in the following experiments were taken from the laboratory stock. The various dysentery bacilli had been carried upon artificial media for many years. The following tests were performed over a period of 15 months.

Twenty-five clean four ounce bottles were fitted with cotton plugs, and sterilized. In each bottle was placed 100 c.c. of meat

infusion broth adjusted to pH 8.0.

Five of the bottles were inoculated with 0.5 c.c. of a 12-hour broth culture of *B. dysenteriae*-Flexner. A second set of five was inoculated with 0.5 c.c. of a 12-hour broth culture of *B. dysenteriae*-Kruse. The third set of five was inoculated with a 12-hour broth culture of *B. dysenteriae*-Shiga. The fourth set of five was inoculated with a 12-hour broth culture of *B. dysenteriae*-Strong. The remaining bottles of broth were used as controls. Sixteen of the inoculated bottles of broth and four controls were incubated at 35 C. The remaining five bottles were placed at room temperature for 442 days. At the end of 72 hours, 30 days, 100 days and 442 days, one bottle of each strain of *B. dysenteriae* and a control were tested.

The following method was used with each strain and its control. Five c.c. of the inoculated broth, likewise of control was removed from each bottle and added to 20 c.c. of sterile meat infusion broth adjusted to pH 8.0. This diluted broth was then filtered. Five c.c. of the sterile filtrate was then inoculated with 0.01 c.c. of a 12-hour broth culture of Flexner, Kruse, Shiga and Strong, respectively. All tubes were incubated at 35 C. and observed at 12 hours and 24 hours, when final readings were made. The results showed no inhibition in any of the tubes.

Agar slants streaked from the tubes after 12 hours' incubation showed normal growth of the test organism.

Since positive results were not obtained in this case, a second test was performed to confirm the absence of lytic agent in the old cultures.

The inoculated filtrates from the preceding test were diluted with 20 c.c. of meat infusion broth and filtered. Five c.c. of the filtrate was then inoculated with 0.01 c.c. of a 12-hour broth culture of the above mentioned organisms. The tubes were incubated at 35 C. At intervals of 6, 12 and 24 hours the tubes were observed, No inhibition was noted in any of the tubes.

Following an incubation period of 12 hours a dilution series of three tubes was made from each inoculated tube of the set-up; by transferring one loop from the tube to a tube of broth, then one loop of this was added to another tube of broth, and so on.

In addition four loops from each of the original inoculated tubes were streaked over the surface of agar slants. At the end of a 24-hour incubation period the agar slants and dilution series tubes were examined.

In this test no inhibition was observed in any of the tubes of the original set-up. Moreover, no lytic areas were observed on the surface of the slants. Furthermore, no inhibition was noted in any of the tubes of the dilution series. The old cultures in every instance failed to generate "spontaneously" a lytic agent.

Next the five bottles of Set I that had been incubated for 72 hours were placed at room temperature. Thus sets of bottles submitted to three growth conditions were obtained; First, the set which was incubated the total time; second, one that was incubated for 72 hours only; and, third, one which had received no incubation, but which was kept from the beginning at room temperature.

At the end of 30 days' incubation the second set of broth cultures was examined for the presence of lytic agent by the method previously employed.

Negative results were obtained.

The third set of five bottles of broth cultures were examined after a 100-day incubation period. The results were also negative.

Finally, after 442 days of incubation at 35 C., the fourth set was examined; also the set that had been incubated for 72 hours, and which thereafter was allowed to remain at room temperature; also the set that had remained at room temperature from the beginning. The same method of procedure as employed heretofore was used. The results were negative in all cases.

From the results of the foregoing tests it was concluded: First, that no lytic agent was present in the original stock cultures. Secondly, that no lytic agent was developed from the ageing of the bacteria themselves.

VI. OCCURRENCE OF THE LYTIC AGENT IN NATURE

Hankin^{13a} reported that the water from certain streams of India were bactericidal for bacteria especially cholera vibrio. He believed this property to be due to the presence of volatile acids. He was unable to completely destroy the bactericidal power of the water by heating in an autoclave at 115 C. for 30 minutes. d'Herelle^{1c} has intimated that this bactericidal activity might have been due to the bacteriophage.

Dumas⁹ subsequently was able to demonstrate the presence of a lytic agent in the waters of the Seine active against colon and dysentery bacilli. However, other workers^{17, 25}, have explained his results as due to the presence of excreta carrying lytic principle and infected with organisms of the colon-typhoid dysentery group.

1. From Natural Waters

In the further attempt to examine possible source of the lytic agent at different times during the summer of 1922, three samples of water were obtained from the Huron River near Ann Arbor. Sample 1 was taken below the sewer outlet of the city on July 14th. Sample 2 from below the dam but above the sewer outlet on July 26th. Sample 3 was obtained below the bridge, near the island, intermediate between the first and second samples, but above the sewer outlet on the 10th of September.

The following method of procedure was adopted with each sample.

Ten c.c. of fresh water was added to 100 c.c. of sterile meat infusion broth adjusted to pH 8.0. The inoculated broth was incubated at 35 C. for 24 hours. A marked turbidity was imparted to all the broths. The turbid broth was filtered through pumice and then through a Berkefeld N filter to serve Samples 1, 2 and 3.

A 1-100 dilution was made from the sample and an inhibition test was performed against various organisms of the colon-typhoid-dysentery group. Twelve 5 c.c. portions were placed in sterile test tubes. Each tube of broth was inoculated with 0.01 c.c. of a 12-hour broth culture of the organisms listed in Table XII. The tubes were incubated for 48 hours when the final readings were made. The results are recorded in Table XII.

From the results given in Table XII it was clear that an inhibiting agent was present in each of the three samples of Huron River water. Two were active against *B. dysenteriae*-Flexner, Kruse, Shiga and Strong, and one was active against Flexner, Kruse and Shiga strains only.

Table XII

Showing the Result of an Attempt to Isolate
a Lytic Agent from the Huron River Water

Dilution of Filtrates 1:100					
Organism	Age of Culture Inoculated	Amount of Culture	Results		
			Sample 1	Sample 2	Sample 3
B.coli	hr. 12	c.c. 0.01	+	+	trace
B.typhosus-Eberth	12	0.01	+	+	+
B. " -Gay	12	0.01	+	+	+
B.paratyphosus "A"	12	0.01	+	+	+
B. " "B"	12	0.01	+	+	+
B.Enteritidis	12	0.01	+	+	+
B.dysenteriae- Flexner	12	0.01	-	-	-
B.dysenteriae- Kruse	12	0.01	-	-	-
B.dysenteriae- Shiga	12	0.01	-	-	-
B.dysenteriae- Strong	12	0.01	-	+	-

Note: + means no inhibition, trace = a trace, and
- complete inhibition.

In order to confirm these results, the tubes of each sample that showed inhibition were pooled, filtered and again tested. A 1-1000 dilution was made from each of the filtrates, in sterile meat infusion broth. The same quantities and method of procedure were used as in the preceding test. All tubes of the set-up were incubated at 35 C. for 48 hours when the final readings were made and recorded as in Table XIII.

Table XIII

Confirmatory Test of the Inhibitive Action of
the Huron Filtrates Against the Organisms Indicated

(Dilution of Filtrates 1:1000)

Organism	Age of Culture	Amount of Culture	Results		
			Sample 1	Sample 2	Sample 3
B. Coli	hr. 12	c.c. 0.01	+	+	+
B. dysenteriae- Flexner	12	0.01	-	-	-
B. dysenteriae- Kruse	12	0.01	-	-	-
B. dysenteriae- Shiga	12	0.01	-	-	-
B. dysenteriae- Strong	12	0.01	-	-	-

From the results presented in Table XIII it is clear that there was present in the Huron River water an inhibiting agent active against B. dysenteriae-Flexner, Kruse, Shiga and Strong in a dilution of at least 1:1000.

In addition, agar slants were inoculated from all the tubes of the set-up. After incubation for 24 hours, Sample 1 tubes from all the strains showed very small pitted areas. Near the edges of the slants the growth was ragged and moth-eaten. In Sample 2 tubes small lytic areas were noted on the Flexner and Kruse slants, while with B. Shiga only ragged edges were noted remaining on the surface of the slants. In Sample 3 tubes all slants of Tube 1 showed many small pitted areas on the surface. The other slants appeared to be normal.

It was concluded from these results that there was present in all samples a lytic agent or

bacteriophage active against the group of dysentery bacilli, namely, Flexner, Kruse, Shiga and Strong.

Further studies were made of the type of colonies produced by the action of the lytic agent on the four strains of dysentery bacilli. Each strain produced the usual two distinct types of colony: one which was round and typical; the other which was irregular with ragged edges and having a granular appearance. Both types were comparable with those obtained from action of the "Hog" filtrates and "Rabbit" filtrate on the sensitive cultures.

Titration were made of the original inhibiting power of each filtrate. Strains No's 1 and 2 failed to inhibit beyond 10^{-3} and strain 3 beyond 10^{-4} using *B. dysenteriae* for the sensitive culture. The three filtrates were next fed young cultures of Shiga bacilli at intervals over a period of 14 months, when a second titration was performed. At this time filtrate 1 had been carried through fourteen successive feedings and filtrations, No. 2 through 15 and No. 3 through 14. A titer of 10^{-7} was obtained for strain No. 1; 10^{-7} for No. 2; and 10^{-8} for No. 3. As a rule the maximum observed titer was obtained after 12 feedings.

2. From Sewage

In further study of sources of the lytic principle samples were obtained from the Imhoff tank and contact bed of the Sewage Disposal Plant at Eloise, Michigan, during the later part of May, 1922.

Five c.c. of each affluent was added to 50 c.c. meat infusion broth adjusted to pH 8.0. The two inoculated broths were incubated at 35 C. for 24 hours. A marked turbidity was noted in both flasks. Ten c.c. of each turbid broth was added to

50 c.c. of meat infusion broth and filtered. The two filtrates were designated as "Imhoff" and "Contact."

The following method of testing inhibitive action was used.

Ten 5 c.c. portions of each filtrate were placed in sterile test tubes. Nine tubes of each filtrate were inoculated with 0.01 c.c. of a 12-hour broth culture of the organisms listed.

The tubes were incubated at 35 C. and the final readings recorded at the end of 48 hours. The results are shown in Table XIV.

Table XIV

Showing the Action of the Filtrates of Imhoff Tank and Contact Bed Against the Organisms Listed:

Organism	Age of Culture	Amount of Culture	Results	
			Imhoff	Contact
B. Coli	hr. 12	c.c. 0.01	Slight inhibition	A trace of inhibition
B. typhosus-Eberth	12	0.01	No "	No "
B. " -Gay	12	0.01	No "	No "
B. paratyphosus "A"	12	0.01	No "	No "
B. " "B"	12	0.01	No "	No "
B. dysenteriae-Flexner	12	0.01	Complete	Complete
B. dysenteriae-Kruse	12	0.01	"	"
B. dysenteriae-Shiga	12	0.01	"	"
B. dysenteriae-Strong	12	0.01	"	"

From the results reported in Table XIV it was apparent that there was present in the "Imhoff" filtrate an inhibiting agent which completely inhibited *B. dysenteriae*-Flexner, Kruse, Shiga and Strong, and which possessed a slight inhibitive action against *B. coli*. However, the "Contact" filtrate completely inhibited all four dysenteriae strains, although it showed only a trace of inhibition against *B. coli*.

Further experiments were performed with the "Imhoff" and "Contact" filtrates in order to increase its potency.

The "Imhoff" tubes showing inhibition were pooled and diluted with five times their volume of meat infusion broth. After thoroughly mixing 50 c.c. of the mixture was filtered. Six 5 c.c. portions of the sterile filtrate were placed in sterile test tubes and five of these were inoculated with 0.1 c.c. of a broth culture of the four dysentery strains and *B. coli*. At the end of a 24 hours' incubation it was noted that all tubes showed complete inhibition.

The same procedure was carried out for the "Contact" filtrate and similar results were obtained.

In addition, the agar slants inoculated from Tubes No's 1 and 2 of each transmission series involving the dysentery bacilli showed many small lytic areas. In the case of *B. coli* there were scattered colonies which appeared to be of the usual two types.

Next, the original maximum inhibiting power of "Imhoff" and "Contact" filtrates, preliminary to feeding, was ascertained.

A series of seven dilutions was made from the same filtrate used in the foregoing test. Each tube was inoculated with one loop of a 12-hour broth

culture of Shiga bacilli. The tubes were then incubated at 37 C. for 48 hours. Readings were taken after the intervals noted in Table XV which presents the details of the test.

From the data presented in Table XV it appeared that the original inhibition titer for the "Contact" and "Imhoff" filtrates against *B. dysenteriae*-Shiga was 10^{-3} .

Next, attempts were made to increase the potency of the two filtrates by the feeding method. At intervals during the process a titration of the lytic value was performed. The results of some of the titrations for "Imhoff" and "Contact" filtrates are recorded in Table XVI.

From these results it is apparent that the two lytic agents possessed similar action. The original titer was the same in both cases and the rate of increase in potency was practically the same. Moreover, the last titrations differed by only one decimal place. It is shown clearly that no increase in inhibition power resulted by further feedings after the thirteenth, at which point the maximum was obtained. Furthermore, both strains produced the same typical colonies with the Dysentery bacilli.

VII. INCIDENTAL TESTS AND OBSERVATIONS

1. Adsorption of the Lytic Agent on Glass

Gratia and de Kruif¹² (1923) using 5 c.c. volumes in their dilution series observed that the maximum inhibition titer of their lytic agent against *B. coli* was 10^{-8} . When, however, they made another similar dilution and then from tube 10^{-8} increased the dilution by adding broth, 1 c.c. (10^{-8} dilution) plus 9 c.c. broth, 1 c.c. (10^{-8}) plus 99 c.c. broth and 1 c.c. (10^{-8}) plus 999 c.c.

Table XV
Titration of the Original Inhibition Value of the
"Contact" and "Imhoff" Filtrates

Set-up				Results								
Strain	Time	Broth Control	L.P. Control	B. Shiga Control	Dilutions							
					10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	
"Contact"	start	-	-	-	-	-	-	-	-	-	-	-
	3 hrs.	-	-	trace	-	-	-	trace	trace	trace	trace	trace
	6 "	-	-	+	-	-	trace	+	+	+	+	+
	9 "	-	-	++	-	-	+	+	+	+	+	+
	12 "	-	-	+++	-	-	++	++	++	++	++	++
	24 "	-	-	++++	-	-	+++	+++	+++	+++	+++	+++
48 "	-	-	++++	-	-	-	++++	++++	++++	++++	++++	
"Imhoff"	start	-	-	-	-	-	-	-	-	-	-	-
	3 hrs.	-	-	trace	-	-	-	trace	trace	trace	trace	trace
	6 "	-	-	+	-	-	+	+	+	+	+	+
	9 "	-	-	++	-	-	+	+	+	+	+	+
	12 "	-	-	+++	-	-	++	++	++	++	++	++
	24 "	-	-	++++	-	-	+++	+++	+++	+++	+++	+++
48 "	-	-	++++	-	-	-	++++	++++	++++	++++	++++	

broth giving final dilutions respectively of 10^{-9} , 10^{-10} , 10^{-11} , and inoculated with *B. coli*, complete inhibition took place in all instances including 10^{-11} . Serial dilutions were made from 10^{-7} and 10^{-6} , and similar results were obtained.

They next prepared a liter of 10^{-9} dilution of the lytic agent with broth and inoculated with sufficient *B. coli*. They next removed 1 c.c., 10 c.c., and 100 c.c. portions and placed them in suitable sterile containers. Following an incubation period the 1 c.c. portion possessed a normal growth of the test organism; the 10 c.c. a slightly less, the 100 c.c. only a trace and the remainder in the flask no growth. Gratia and de Kruif concluded from these results that the lytic agent had been absorbed by the glass of the tubes and smaller flasks.

The writer who repeated this experiment was able to obtain similar results using a lytic agent active against *B. coli*; and whose maximum inhibition titer was also 10^{-8} .

These confirmed results led the author to ascertain whether some other method of increasing the glass surface exposed to the inoculated broth would change the titer of the lytic agent. The glass surface in contact with the broth in each tube was increased by the use of glass beads from about 11.7 sq. c.m. to 47.36 sq. c.m. A titration was performed using the anti-*coli* lytic agent and a sensitive strain of *B. coli*. An inhibition titer of 10^{-8} was obtained. A control titration using the same lytic agent and *B. coli*, without the glass beads gave the same inhibition titer. It was concluded from the results obtained that the increase in surface caused no loss of lytic power through an assumed adsorption of the lytic agent upon the glass. It seems reasonable to believe that the results obtained by Gratia and de Kruif must have some other explanation than the glass adsorption hypothesis.

2. Effect of Distilled Water Upon the Lytic Agent

Further studies were performed upon the lytic agent and *B. coli* in an attempt to determine the effect of sterile distilled water upon the action of the lytic agent. Dilutions of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , and 10^{-10} were made in sterile distilled water. The inhibition titer of each dilution was then determined as follows: 0.5 c.c. of each aqueous dilution was further diluted with 4.5 c.c. sterile broth making a 10^{-1} dilution. From this tube a series of dilutions were made, each increasing by 10. Each tube was then inoculated with one loop of a 12-hour broth culture of a sensitive strain of *B. coli*. Controls for the lytic agent in broth and organism were included in the set-up. Final readings were taken at the end of a 48 hours' incubation at 35 C. The results are shown in Table XVII.

It appears from the results expressed in Table XVII that the procedure of making a portion of the dilutions in distilled water had no influence on the final inhibition titer of the lytic agent. In all cases this was 10^{-8} regardless of the relative number of preliminary dilutions made in distilled water. It thus appeared that, during the time of the experiment, the contact of lytic agent with distilled water produced no flocculation of the principle and no observed diminution in the energy of action.

Table XVII
Summary of Titrations of the Inhibition Power
of Dilutions of the Anti-Colon Lytic Agent with Sterile
Distilled Water

Titrations	Lytic Agent Broth Control	B. Coli Control	Results									
			10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰
Control	-	+	-	-	-	-	-	-	-	-	+	+
10 ⁻¹ (H ₂ O)	-	+	-	-	-	-	-	-	-	+	+	+
10 ⁻² "	-	+	-	-	-	-	-	-	+	+	+	0
10 ⁻³ "	-	+	-	-	-	-	-	+	+	+	0	0
10 ⁻⁴ "	-	+	-	-	-	-	+	+	+	0	0	0
10 ⁻⁵ "	-	+	-	-	-	+	+	+	+	0	0	0
10 ⁻⁶ "	-	+	-	-	+	+	+	+	0	0	0	0
10 ⁻⁷ "	-	+	-	+	+	+	+	+	0	0	0	0
10 ⁻⁸ "	-	+	+	+	+	+	+	0	0	0	0	0
10 ⁻⁹ "	-	+	+	+	+	+	0	0	0	0	0	0
10 ⁻¹⁰ "	-	+	+	+	+	+	0	0	0	0	0	0

- = complete inhibition, + no inhibition, and 0 no test made.

VIII. SUMMARY

This paper deals with a study of the source of the lytic agent. Isolations of bacteriophage were attempted from normal tissues, embryonic tissues, pathological tissues, natural waters and from sewage.

Of the intestines of six hogs examined a lytic agent was isolated in two cases. The two agents were similar in action and active against *B. dysenteriae*-Flexner.

Of 24 normal rabbit intestines examined a lytic agent was in no case obtained; neither was one obtained from the intestines of four rabbits immunized against Flexner bacilli. In the case of a rabbit however, which had been immunized against human blood serum a lytic agent active against *B. dysenteriae* Shiga was obtained; also from a spontaneous abscess a lytic agent active against *Pasteurella cuniculicida* was observed.

Of 12 normal guinea-pig intestines examined a lytic principle was in no case obtained.

From embryonic tissue of chicks (25 cases), guinea pigs (8 cases), rabbits (34 cases), and rats (24 cases), no lytic principle was obtained active against Shiga.

In three attempts to isolate a lytic agent from the Huron River water near the city of Ann Arbor, positive results were obtained in every case. The bacteriophage was active against Flexner, Kruse, Shiga and Strong bacilli.

In one examination of material from an Imhoff tank and in one examination from a Contact bed a lytic agent was isolated active against the four dysentery strains and also against *B. coli*.

In all cases the activity of the original lytic filtrates could be intensified, by the "feeding method." The maximum inhibition titer secured for the various lytic agents was-

1.	Hog filtrate No. 5	10 ⁻⁷
2.	Hog " " 6	10 ⁻⁸
3.	Rabbit - " 1	10 ⁻⁸
4.	Rabbit abscess filtrate	10 ⁻⁶
5.	Huron filtrate No. 1	10 ⁻⁷
6.	Huron " " 2	10 ⁻⁷
7.	Huron " " 3	10 ⁻⁸
8.	Imhoff "	10 ⁻⁷
9.	Contact "	10 ⁻⁸

In the attempts to increase the inhibition titer by feeding it appeared that in general, the maximum titer was obtained after 12 feedings. Each bacteriophage reached a dilution limit of active inhibition.

A typical "suicide culture" of *Pasteurella cuniculicida* was obtained from a spontaneous abscess on a rabbit and this culture is described.

In all cases the action of the lytic principle upon a sensitive culture resulted in the production of a resistant type differing from the sensitive in many respects.

Attempts to isolate a lytic agent "from the bacteria themselves", when the cultures were incubated over a long period of time, were in every instance negative.

Incidentally an attempt was made to confirm the surface adsorption test of Gratia and de Kruif, increased surface exposed to the lytic agent being obtained by the use of glass beads. No evidence was gained that the diminution of activity of the lytic principle was caused as a result of the increase in surface.

Whether the preliminary dilutions of the lytic filtrate, used for inhibition tests, were made in distilled water or in broth made no difference in the final titer as shown by the ultimate dilutions made in broth. In all cases the final inhibition titer stood at 10⁻⁸. It thus appeared that distilled water, acting on the lytic agent for

periods of one to four hours, did not result, either in the enhancement or in the attenuation of lytic power.

The conclusions reached from the study of source were that the lytic agent can easily be isolated from sewage infected water supplies; that it is common in pathological tissues, but absent from embryonic and normal adult tissue. Moreover, that it is not formed from prolonged contact between embryonic tissue and bacterial cells. Furthermore, that the inhibitive and germicidal action sometimes observed in normal tissue is due to some other agency than the lytic agent.

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